Supplemental Data

Safety and efficacy of anti-human activated protein C antibody SR604 for prophylaxis of congenital factor deficiencies

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Supplemental materials and methods

Affinity maturation and molecular characterization

Homology modeling was carried out on murine VH/VL sequences of HAPC1573, and the optimal human germline gene sequences were obtained. After comparing APC binding abilities of various humanized HAPC1573 constructs expressed and the numbers of back mutations introduced, one construct (Humab-14) with the highest binding ability towards APC and least back mutation was selected for subsequent affinity maturation. Each amino acid of six complementarity-determining regions (CDRs) of Humab-14 Fab was respectively mutated into 19 different amino acids by site-directed mutagenesis. The improved APC binding mutants with unique Fab sequences were ranked based on results of antigen-coated direct ELISA. Those point mutations in the VH and VL determined to be beneficial for antigen binding were further combined for additional binding synergy. A combinatorial construction was carried out, and the sequences were constructed into a whole IgG form for expression and purification. Based on the expression level and antigen binding affinity, SR604, a humanized IgG4 with four amino acid mutations in CDRs compared to Humab-14, was selected for further characterization. Computation-based structural simulation including modeling, docking, binding affinity, and protein interaction analysis was performed using Bioluminate (Suites 2022–3, Schrödinger, Inc., New York). SR604 Fab and Humab-14 Fab were homology modeled based on sequences and structural alignment to PDB 6M3C, which is the atomic coordinates and structure factors of APC and HAPC1573Fab complex structure deposited in the Protein Data Bank (wwpdb.org). APC-SR604 Fab and APC-Humab-14 Fab models were predicted by the Protein-Protein Docking Module with restraints of interactions of HAPC1573 and APC from PDB 6M3C. Mutation-mediated changes within SR604 Fab and Humab-14 Fab binding to APC were calculated by the Residue Scanning Calculation Module. Surface complementarity and protein–protein interaction between SR604 Fab/Humab-14 Fab and APC were calculated by the Protein Interaction Analysis module. The ribbon and surface maps were illustrated by Pymol (Schrödinger, Inc., New York).

Protac-induced protein C activation in activated partial thromboplastin time (aPTT) assay

Protac (Pentapharm; New Jersey, USA; 113-01) at 0.2, 0.2, 0.05, 0.1, 0.05, 0.1, 0.05, or 0.4 U/mL was added to the following human plasma samples: FV-deficient plasma (IL; REF 20011500), FVII-deficient plasma (IL; REF 20011700), FVIII-deficient plasma (IL; REF 20011800), FIX-deficient plasma (IL; REF 20011900), FX-deficient plasma (IL; REF

20010000), FXI-deficient plasma (IL; REF 20011300), VWF-deficient plasma (Boatman Biotech; REF BMC18), and calibration plasma (IL; REF 20003700). Various concentrations of SR604 were incubated with the mixture at room temperature for 15 min, and 50 µl of the reactions were subsequently incubated with aPTT reagent (50 µl; IL; REF 20006800) at 37°C for 180-220 s. Then, 20 mM $CaCl_2$ (50 µl; IL; REF 20006800) was added, and the clotting time was recorded by an automated analyzer (ACL TOP 750, IL) and plotted against the various SR604 concentrations.

Blood tests

Eight-week-old male *PROC*$^{+/+}$*;F8*$^{-/-}$ and *PROC*$^{+/+}$*;F9*$^{-/-}$ mice received antibody and LPS as described above. Eighteen hours after LPS injection, whole blood was collected from the inferior vena cava into 3.8% sodium citrate and centrifuged for plasma preparation. Plasma levels of ALT, AST, LDH, and IL6 were measured.

Histological analysis

Fourteen days after the knee injury, the mice were sacrificed, and the knee joints (femur and tibia/fibula regions, 1 cm from each direction of the joint) were harvested and treated with 10% formalin fixative. After 24 hours of fixation, the knee joints were decalcified for seven days, then processed in graded alcohol, and embedded in paraffin. The tissues were cut into thin sections of 5-8 um, and stained with hematoxylin and eosin (H&E).

For analysis of tissue histology after sublethal challenge of LPS, lungs were fixed overnight in 10% formalin at 4°C. After being rinsed in PBS three times for 30 min/time, the tissues were dehydrated in an automatic dehydration apparatus, embedded in paraffin, and cut into 6 µm-thick sections for H&E staining.

Immunofluorescence staining and confocal microscopy imaging

Tissues were harvested and postfixed in freshly prepared 4% PFA in PBS overnight at 4˚C. After washed in PBS for 3 x 30 mins, tissues were cryopreserved in 20% sucrose overnight at 4˚ and then embedded in OCT. OCT blocks were sectioned t at a thickness of 20 µm. Sections were mounted onto slides, and then incubation in blocking buffer having 3% BSA (Sigma-A9647), 5% goat serum, 5% donkey serum, 0.3% Triton X-100 (Thermo Fisher Scientific -85111) in PBS for 3 hours at room temperature. Afterward, the sections were incubated with primary antibodies, i.e., armenian hamster anti-mouse CD31 (Chemicon International, MAB1398Z), rabbit polyclonal to CD45 (abcam, ab154885). in blocking buffer diluted in PBS (1:2) overnight at 4˚C.

After washing 2 x 10 mins in 0.1% Triton X-100 and then PBS, secondary antibodies conjugated with Alexa Fluor [568, goat Anti-Armenian hamster IgG H&L (abcam ab175716), 488, or goat anti-rabbit IgG H&L (Abcam, ab150077) were added at 5 μg/ml in PBS. 3 x 15 mins washes were followed with PBS. Tissue sections were then stained with DAPI (Biolegend-422801) for 15 mins followed by a quick wash with PBS and then mounting with fluoromount aqueous mounting medium (SA-F4680). Imaging was taken and analyzed by a confocal microscope (Leica Microscope System SP8, 40x/0.95 Plan Apochromat objective).

SDS-PAGE analysis of APC +/- SR604 cleaving histones

Eight samples were prepared as following: 0.2 mg/ml calf thymus histones (Shanghai yuanye Bio-Technology Co., Ltd.), 200 nM HAPC (Prolytix, HCAPC-0080), 400 nM SR604, 400 nM IgG4 0.2 mg/ml, histones + 200 nM HAPC, 0.2 mg/ml histones + 400 nM SR604, 0.2 mg/ml histones + 200 nM HAPC + 400 nM SR604, 0.2 mg/ml histones + 200 nM HAPC + 400nM IgG4, respectively, in PBS buffer. After mixing, samples were placed in 37℃ water bath for 1 hour. Then 50 μl samples plus 50 μl non-reducing loading buffer (Beijing BioRab Technology Co. Ltd.) were boiled for 8 minutes. After boiling, 50 μl sample was used for SDS-PAGE (12% gel, GenScript) analysis.

Endothelial cell permeability

Endothelial permeability was measured by the flux of Evans blue dye across an endothelial monolayer. Briefly, human umbilical endothelial cells (HUVECs) were seeded in transwells of 3 μm pore size and 12 mm diameter (Corning, item number 3462) for 48 h. The cells were incubated with 20 nM APC with or without 100 nM SR604 or human IgG4 isotype mAb (Bei Jing SinoBiological, item number HG4K) in endothelial cell medium (ECM) containing 0.4% BSA for 3 hours and then replaced with ECM containing 0.4% BSA and 5 nM bovine thrombin for 10 min. The upper chamber was replaced with ECM containing 0.4% BSA and 0.67 mg/ml Evans blue and the lower chamber was replaced with ECM containing 0.4% BSA. The Evans blue media in the lower chamber was measured for OD 650 after 2 min incubation.

Acute Toxicity

PROC$^{+/+}$*;F8*$^{-/-}$ and *PROC*$^{+/+}$*;F9*$^{-/-}$ male mice were used for this experiment. Mice were randomly divided into a negative control group (five mice) and a treated group (five mice). The negative control group was given SR604 formulation buffer. To evaluate the potential toxicity of SR604, a 1,000-times higher dose (100 mg/kg) than the proposed human clinical trial dose of SR604 (0.1

mg/kg subcutaneously) was subcutaneously administered into the treated group. After treatment, mice were closely monitored for clinical signs including appearance, activity, coat, urine and feces, body weight changes, and mortality for 14 days. At the end of experiments, peripheral blood samples were collected for routine blood cell counts and blood chemistry analysis (ALT, AST, LDH, etc.). Histology of sacrificed mice was also performed at the end of experiments.

Supplemental Table 1. Amino acid sequences of murine HAPC1573 Fab, humanized Humab-14 Fab, and the SR604 humanized chimeric antibody.

Antibody	Region	Isotype	Amino acid sequence
HAPC1573 Fab	V_K	Murine kappa	NIVLTQSPASLAVSLGQRATISCRASESVDSFGATFMHWYQQKPGQ PPKLLIYLASNLESGVPARFSGSGSRTDFTLTIDPVEADDAATYYCQQ NNEDPYTFGGGTKLEIK
	V_H	Murine IgG1	EVKLEESGGGLVQPGGSMKLSCVASGFTFSNYYLNWVRQSPEKGL EWVADIRLKSNNYEKHYAESVKGRFTISRDDSKSSVYLQMNNLRAE DTGIYYCIREGDYFDYWGQGTTLTVSS
Humab-14 Fab	V_K	Human kappa	DIQLTQSPSSLSASVGDRVTITCRASESVDSFGATFMHWYQQKPGK APKLLIYLASNLESGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQ NNEDPYTFGQGTKLEIK
	V_H	Human IgG1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYLNWVRQAPGKGL EWVGDIRLKSNNYEKHYAESVKGRFTISRDDSKSITYLQMNSLRAED TAVYYCAREGDYFDYWGQGTTVTVSS
SR604 antibody	Light Chain	Human kappa	DIQLTQSPSSLSASVGDRVTITCRASESVDSFGATFMHWYQQKPGK APKLLIYLASRLGSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQ NNEDPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLN NFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK ADYEKHKVYACEVTHQGLSSPVTKSFNRGEC
	Heavy Chain	Human IgG4	EVQLVESGGGLVQPGGSLRLSCAASGFTFSFYYLNWVRQAPGKGL EWVGDIRLKKNNYEKHYAESVKGRFTISRDDSKSITYLQMNSLRAED TAVYYCAREGDYFDYWGQGTTVTVSSASTKGPSVFPLAPCSRSTSE STAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVESKYGPPCPPCPAPE FLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYV DGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK

Underlines: CDRs; Red, mutated amino acids. Light blue, C_K. Blue, C_H

Supplemental Table 2. Calculated binding affinity (to APC) and stability (in kcal/mol) of HAPC1573 variants bearing one or all of the four mutations from Humab-14 to SR604.

Residue	Original	Mutated	d Affinity	d Stability (solvated)
H:31	ASN	PHE	-5.24	-4.43
H:55	SER	LYS	-6.19	-0.76
L:57	ASN	ARG	-20.61	-26.46
L:59	GLU	GLY	-1.65	8.83
H:31	ASN	PHE	-31.46	-21.33
H:55	SER	LYS		
L:57	ASN	ARG		
L:59	GLU	GLY		

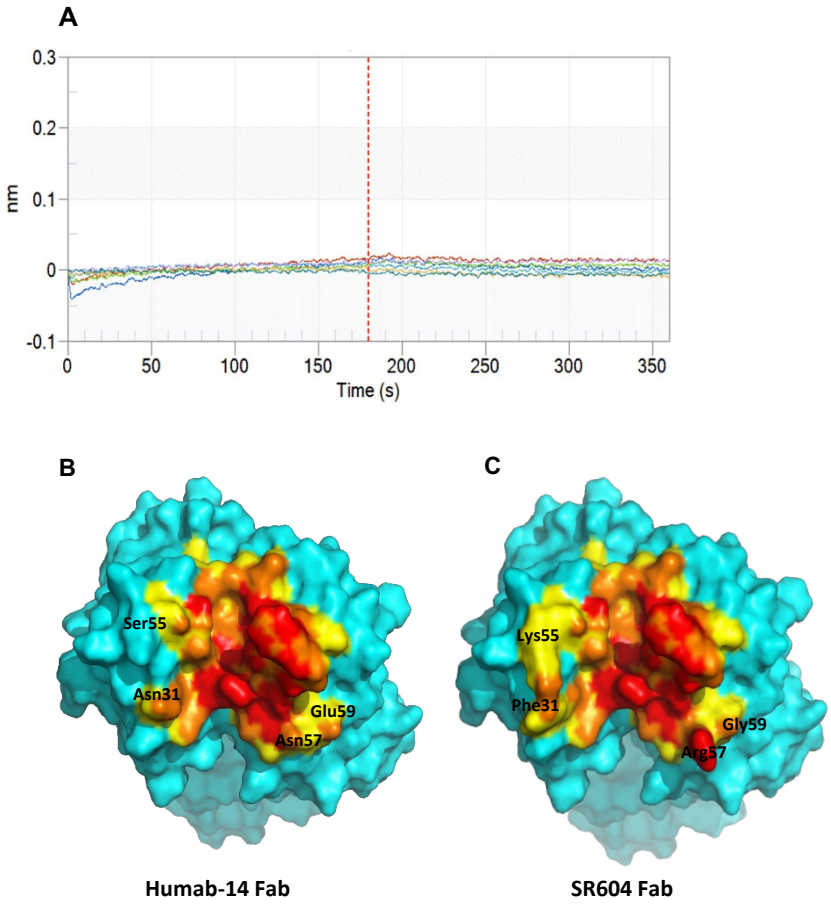
APC-binding affinity were calculated by the Residue Scanning Calculation Module of Bioluminate, Schrödinger Suites 2022–3. H; heavy chain; L: light chain.

Supplemental Table 3. Calculated surface complementarity and molecular distance between APC and Humab-14 Fab or SR604 Fab.

Residues in Fab		Residues in hAPC	Distance (A)	Specific interactions	# H bond	# Salt bridges	Surface complement -arity
Residues in Humab-14	L:Asn 57	A:His 430	3.2		0	0	0.88
	L:Asn 57	A:Ser 378	3.5		0	0	0
	H:Asn 31	A:Arg 394	3.6		0	0	0.53
	H:Asn 31	A:Leu 429	3		0	0	0.53
	H:Asn 31	A:Gly 392	3.2		0	0	0.52
	H:Asn 31	A:Asp 393	3.3		0	0	0
Residues in SR604	L:Arg 57	A:Ser 378	2.2		0	0	0.7
	L:Arg 57	A:Val 376	2.7		0	0	0.75
	L:Arg 57	A:Met 377	2.2		0	0	0.7
	L:Arg 57	A:Glu 424	2.4	1x h bond, 1x salt bridge to A:Glu 424	1	1	0.71
	L:Arg 57	A:Asn 431	3		0	0	0.51
	L:Arg 57	A:His 430	3.4		0	0	0.79
	L:Arg 57	A:Glu 375	3.6		0	0	0
	H:Lys 55	A:Asp 214	2.9	1x salt bridge to A:Asp 214	0	1	0
	H:Phe 31	A:Arg 394	3.8		0	0	0.5

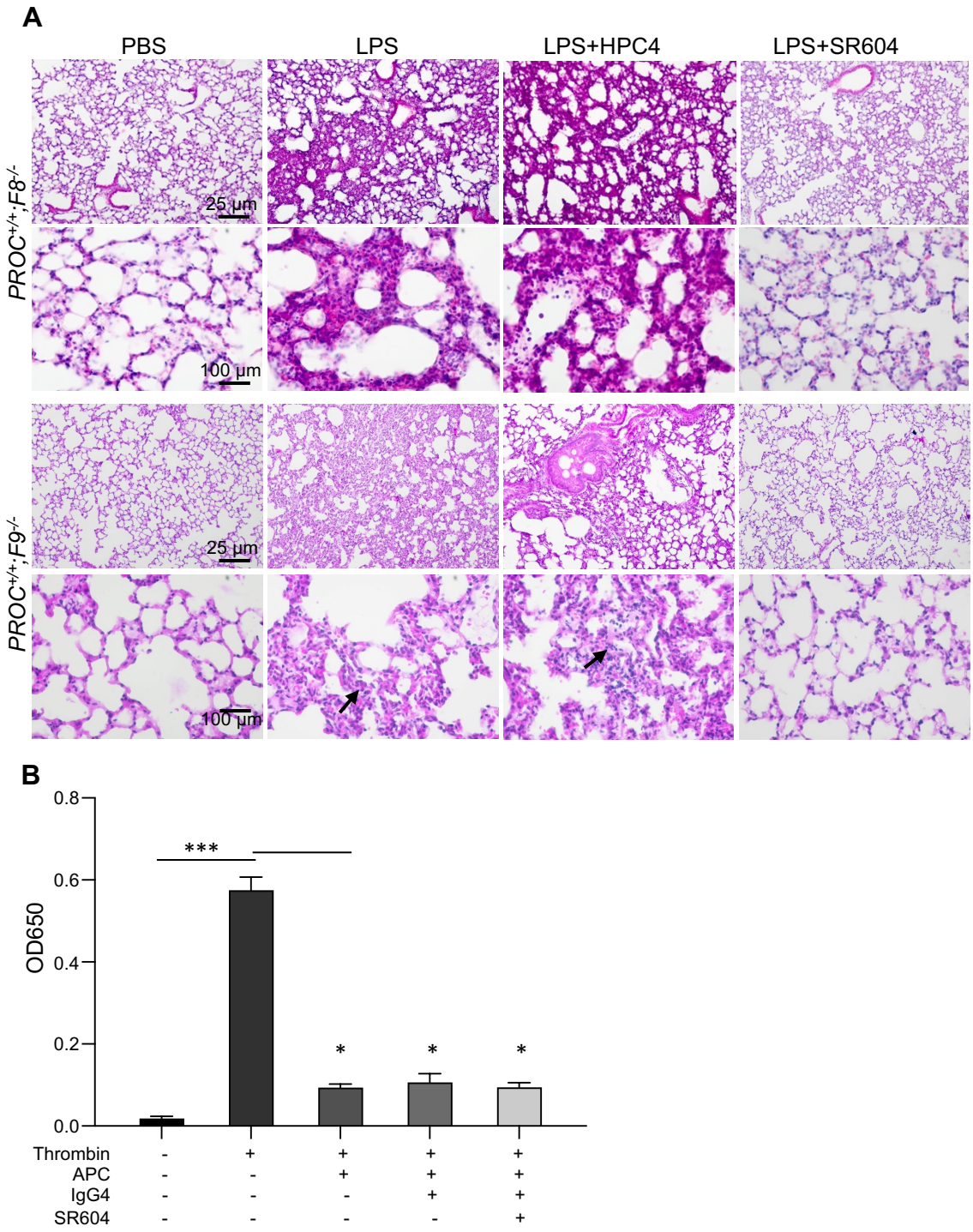
Surface complementarity and molecular distance between APC and Humab-14 Fab or SR604 Fab were calculated by the Protein Interaction Analysis module of Bioluminate.

Figure S1



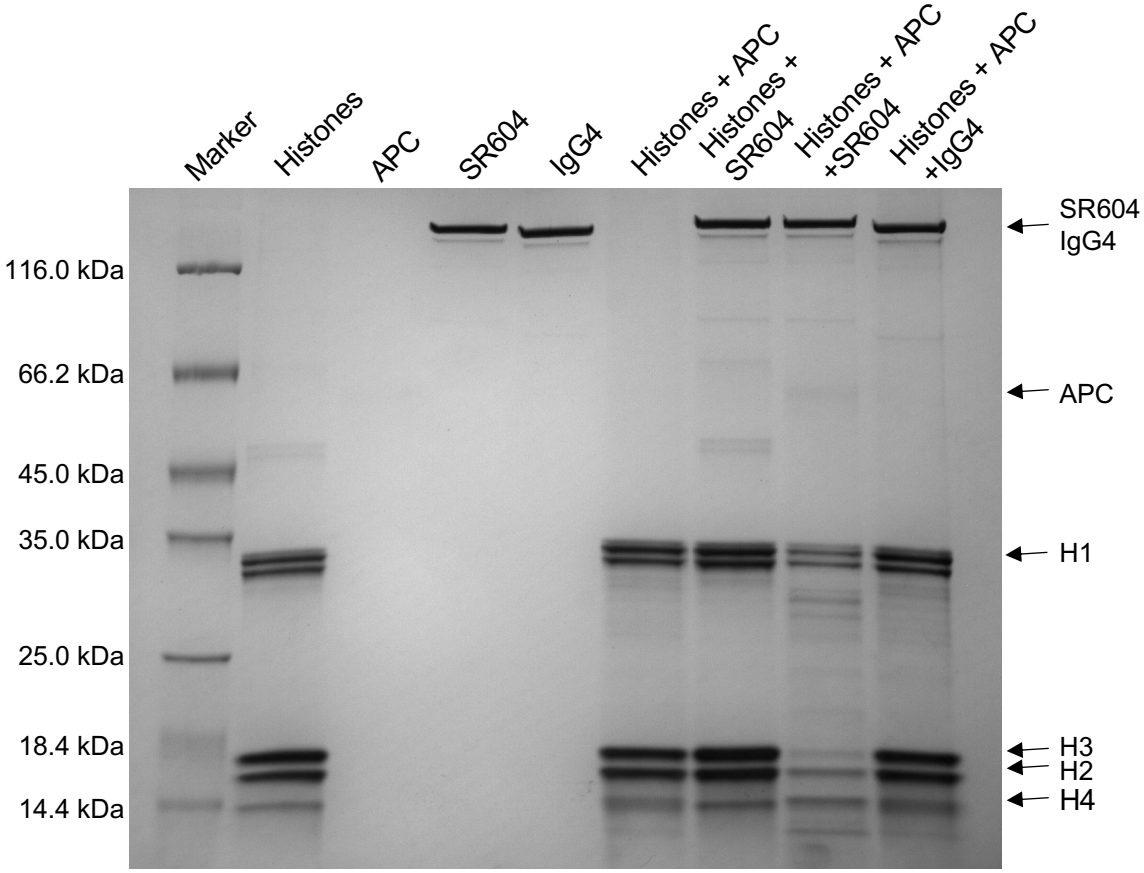
Supplemental Figure 1. A: SR604 does not bind to human plasma protein. BioLayer Interferometry (Octet) method was used to measure the binding of SR604 with human plasma proteins. Briefly, biotinylated SR604 was immobilized onto SA biosensor. Human plasma diluted at 1/25, 1/50, 1/200, 1/400, and 1/800 was analyzed (from the top to the bottom). The association and dissociation times were set to 180 s. The raw data sensorgram (nm) shows that no binding response was detected. **B-C, Interactions of APC with SR604 and Humab-14 Fabs.** Close-up views of the interface between APC and Humab-14 Fab, or APC and SR604 Fab. The four residues that are different between the two Fabs are marked.

Figure S2



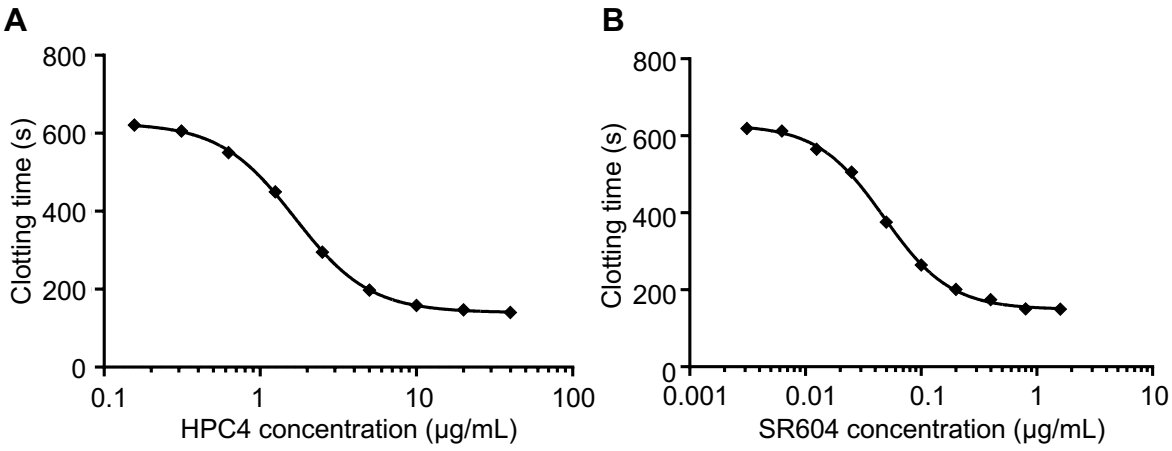
Supplemental Figure 2. (A) Representative images of H&E-stained paraffin sections of lungs. (B) Endothelial permeability was measured with HUVECs in a transwell assay pretreated with APC in the absence or presence of SR604 or Human IgG4 isotype mAb and thrombin-induced flux of vans blue dye across the cell monolayer was measured as described in Materials and Methods. The results shown are the means +/- SEM performed in three replicates.

Figure S3



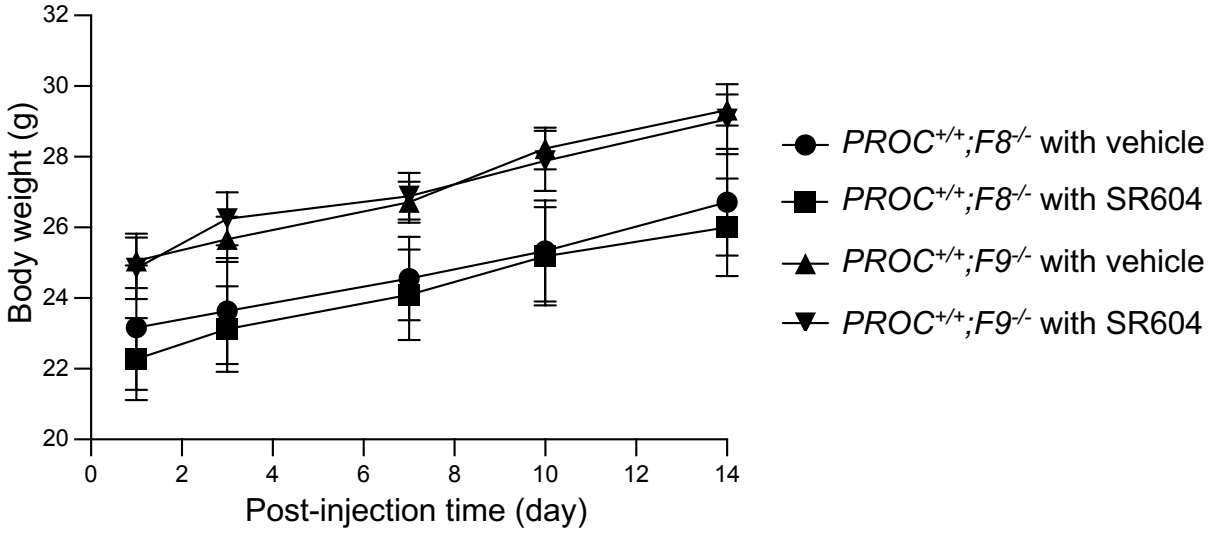
Supplemental Figure 3. SR604 enhances APC cleaving histones. Calf thymus histones were incubated with APC in the presence or absence of SR604 or isotype control antibody at 37℃ for 1 h. Samples were then analyzed by SDS-PAGE under non-reducing condition.

Figure S4



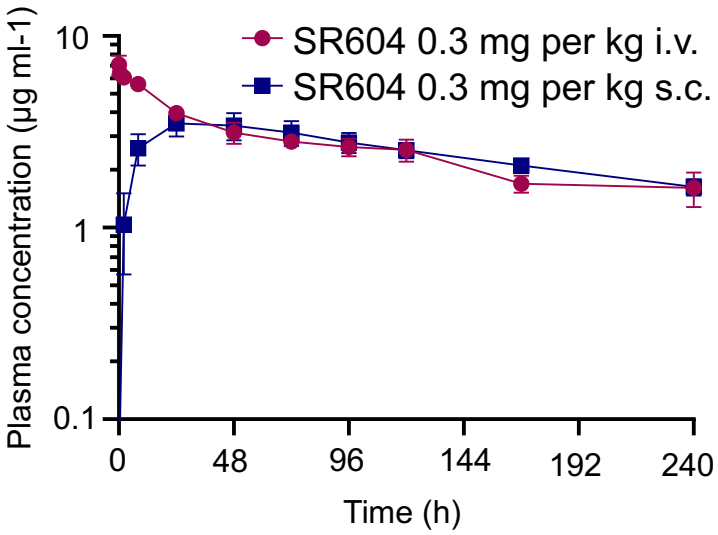
Supplemental Figure 2. HPC4 and SR604 corrected the prolonged clotting time of human FVIII-deficient plasma. Human FVIII-deficient plasma was incubated with 0.15625 - 40 µg/ml of HPC4 (anti-human protein C antibody) or 0.00313-1.6 µg/ml of SR604 in the presence of Protac (0.05 U/ml) for 15 min. The mixtures were then incubated with aPTT reagent for 180 –220 s, and CaCl2 was added to trigger clotting. Clotting times were recorded by an automated analyzer. The data represent two experiments.

Figure S5



Supplemental Figure 3. SR604 does not cause acute toxicity in humanized hemophilia mice. PROC+/+;F8-/- and PROC+/+;F9-/- mice were subcutaneously injected with 100 mg/kg of SR604 or vehicle control and their bodyweights recorded for 14 days after the injection. n = 5

Figure S6



Supplemental Figure 4. SR604 has prolonged half-life in monkeys after injections. Time course of the plasma concentration of SR604 in cynomolgus monkeys after 0.3 mg per kg body weight of intravenous (i.v., n = 3) or subcutaneous (s.c., n = 3) injection. Data are expressed as means ± s.d. Pharmacokinetic parameters were calculated by WinNonlin software. The subcutaneous bioavailability was calculated by dividing the AUCinf for the subcutaneous administration by the AUCinf for intravenous administration.